CHAPTER 6

General discussion

In this thesis, we demonstrate the experimental capacity of one nontraditional model organism: the free-living flatworm *Macrostomum lignano*. The remarkable biological features of *M. lignano*, such as the presence of adult pluripotent stem cells and impressive regeneration capacity combined with an ease of maintenance in the laboratory conditions and constantly expanding toolbox make it scientifically very attractive and worth to explore (Arbore et al., 2015; Ladurner et al., 2000; De Mulder et al., 2009; Pfister et al., 2007; Plusquin et al., 2015; Wudarski et al., 2017).

Characterization of the neoblast population

Stem cell biology, a dynamic area of research, is marked by rapid progress and great discoveries such as the identification of a stem cell niche, reprogramming of somatic cells into pluripotent stem cells or the introduction of organoids (Laita et al., 2003; Sato et al., 2009; Takahashi and Yamanaka, 2006; Williams et al., 1999). It is particularly interesting, and in the same time extremely difficult in traditional model organisms, to study pluripotent stem cells in their natural environment within the physiological context. The field would therefore benefit enormously from expanding the inventory of available experimental models. For instance, it was unambiguously shown that flatworm *Schmidtea mediterranea* possess adult pluripotent stem cells (Wagner et al., 2011). That certainly makes it an interesting model for *in vivo* stem cell studies (Rink, 2013).

In **Chapter 2** we explore the potential of another flatworm, *M. lignano*, to investigate *in vivo* stem cell functioning. We first generated a *de novo* transcriptome assembly of the worm. Next, we used two approaches to identify genes enriched specifically in proliferating cells: (1) comparisons of gene expression in irradiated worms, devoid of proliferating cells, and control worms, and (2) comparison of FACS-isolated differentiated and proliferating cells. Moreover, by isolating cells from adult animals, and from juveniles and amputated heads, which lack gonads, we could distinguish the enrichment of transcripts in the proliferating germline cells and in the somatic neoblasts. To increase the accessibility of the generated transcriptional datasets, we have created an online resource available at http://neoblast.macgenome.org.

We have also performed an RNAi screen, revealing a number of conserved genes crucial for the functionality of somatic neoblasts during homeostasis and regeneration. Notably, *Mlig-ddx39(RNAi)* worms demonstrated the most robust and severe phenotype, and the gene became a reliable, positive control for all ongoing and future knockdown experiments in our laboratory. However, the exact role of *Mlig-ddx39* in the maintenance of the worm's proliferating cells is to be determined. It would also be es-

sential to explore whether the function of *ddx39* gene in the context of stem cells is conserved in higher organisms, including humans.

This study, arguably, lays ground for future experiments. Further exploration of the generated lists is worth pursuing. Investigating functions of other, not yet screened genes holds a promise of identifying other stem cell markers. Knockdown studies could be either performed as described in this thesis, to find robust phenotypes, or alternatively could be expanded to longer runs and more detailed analysis to determine subtle phenotypes (see discussion Chapter 2). Interesting candidates should be followed-up using the traditional models, such as mouse for *in vivo* studies and cell cultures for *in vitro* tests, to investigate the conservation of the gene function.

Furthermore, a comprehensive characterization of the *M. lignano* neoblast population is essential. The here generated datasets and identified markers, side by side with the described markers of planarian neoblast subpopulations (Molinaro and Pearson, 2016; Van Wolfswinkel et al., 2014; Wurtzel et al., 2015), form a convenient basis for further description of the neoblast by e.g. single cell RNA-Seq. The ultimate aim of these studies is the identification of the true pluripotent stem cells, which are still not characterized in any of the flatworm species. Additionally, combination of data presented in this chapter and the recently demonstrated amenability of the worm to transgenesis (Wudarski et al., 2017), can be used to create transgenic lines permitting advanced stem cell analysis and efficient lineage tracing. Consequently, elucidating the mechanism controlling the stem cell lineage commitment in *M. lignano* could shed light on how this process is regulated in human adult stem cells. In summary, the advances in flatworm stem cell biology could provide a long-awaited powerful model for *in vivo* studies.

The value of flatworm-specific genes and their potential in biomedical research

One of the core characteristics of an informative experimental model is the ability to extrapolate results generated using such organisms, to other, often more complex organisms, such as *Homo sapiens*. Therefore, research typically focuses on conserved genes and pathways, to indeed facilitate further generalization of the findings. In contrast, in **Chapter 3,** we describe a study exploring the importance of nonconserved genes. From the available lists of transcripts enriched in proliferating somatic and germline cells (Grudniewska et al., 2016) we have selected several candidates specifically not conserved in human. An initial RNAi screen revealed that one of the novel genes, *Mlig-sperm1*, is required to produce healthy sperm and its knockdown significantly re-

duces fertility of the worms.

Overall, the chapter illustrates the current setup of the experimental screening platform for efficient characterization of the expression pattern and function of potentially interesting genes. So far, we have only looked into a limited number of candidates. The generated lists, however, provide hundreds of them and further efforts on the characterization of these genes are needed. With this pipeline in place, extending the analysis is straightforward. Exploring additional flatworm-specific genes could provide novel insight into and provide potential therapeutic targets for combating infections caused by parasitic species. Furthermore, nonconserved genes, as demonstrated recently (Hashimoto et al., 2016), might have a great biomedical potential. Synthetically produced proteins, derived from regeneration-capable animals could improve wound healing process in human. Genetically engineered cell cultures with the DNA from organisms highly resistant to ionizing radiation could protect human DNA. The above examples illustrate the new era of biomedicine yet to come.

Rejuvenation and ageing

The ultimate goal of longevity research is to understand the mechanisms of ageing and, ideally, to extend the healthspan. Extreme longevity and immortality have captured the attention of researchers for decades (Rando, 2006). An enduring hypothesis of regeneration-induced rejuvenation attracted our attention too. Several exciting reports suggested that multiple amputations in flatworms may lead to lifespan extension and even a reversal of ageing (Egger, 2008; Egger et al., 2006; Haranghy and Balázs, 1964; Lange, 1968). On the other hand, a more recent study focusing on telomere length and telomerase activity in the planarian *Schmidtea mediterranea* indicated that the rejuvenation effects are only observable in the asexual strain, and not the sexual (Tan et al., 2012). The proof for the hypothesis is scarce and presented evidence is rather ambiguous. We have therefore decided to systematically investigate it in *M. lignano*. In **Chapter 4** we describe our efforts to verify whether single and repeated regeneration affect ageing and can cause rejuvenation. Moreover, to get a full picture of the worm's ageing, we studied several parameters, such as morphology, survival, fertility, and gene expression. Although we did not find an evidence of rejuvenation, the study provided a comprehensive ageing profile of *M. lignano*. Interestingly, the majority of genes identified as essential for neoblast functionality in Chapter 2, were upregulated with advancing age, suggesting the importance of stem cell maintenance in ageing.

In the context of that study, the fascinating question of a great significance remains however unanswered. What is the impact of sexual and asexual reproduction on

the animal's lifespan? It comes as no surprise therefore, that in the general context of ageing research, alternative models are needed. The incredible biodiversity of the animal kingdom offers a wide range of organisms: from animals demonstrating extremely short lifespans to exceptionally long-lived species. It is particularly interesting to explore how the latter ones are successfully in resisting the ageing forces. Studying organisms with negligible senescence may provide clues about how to extend human's and animal's healthspan. Comparative biology approaches may yield valuable insight and help to understand observed differences between longevity in different species.

Highly efficient DNA damage control

The genome of eukaryotic cells is constantly exposed to damage, and an efficient DNA repair mechanism is therefore essential for genome integrity (Signer and Morrison, 2013). Its faulty functioning is implicated in cellular ageing and cancer formation (Blasco et al., 2007). It was shown before that *M. lignano* displays remarkable resistance to large doses of ionizing radiation (IR) (De Mulder et al., 2010). However, the precise mechanisms of that high radioresistance are unknown. In **Chapter 5** we confirmed that indeed the worm demonstrates much higher endurance to IR than humans. We also generated a comprehensive transcriptional signature of the early DNA damage response (DDR) to γ-irradiation. The generated gene lists that are enriched for the known DDR genes will serve as a basis for the selection of candidate genes for further studies.

This chapter also describes the efforts to develop a reliable method to quantify DNA damage in *M. lignano* cells. Despite the initial success, further optimization of the comet assay is needed, as, at times, we could not obtain a reliable negative control, suggesting high levels of damage introduced during sample preparation. Furthermore, several alternative paradigms to measure and/or quantify DNA damage and repair should be explored. In addition, as limited changes in DDR genes were observed, a good complementation of the transcriptional profile would be the characterization of the post-irradiation proteasome. Changes in gene expression do not always correspond to alterations on the protein level, and inversely, some protein modifications are not always reflected on gene expression level. Such a comprehensive profile of the DDR would not only add to our understanding of efficient protection and repair mechanisms of flatworm's DNA, but also, following a recently published approach (Hashimoto et al., 2016), could become a source of therapeutic interventions in biomedicine.

Concluding comment: lessons from the nontraditional animal model

From the first pages of this thesis, I aimed to emphasize the importance of nontraditional experimental organisms in modern biology. Certain limitations of commonly used animal models and the increasing availability of research tools which are applicable to diverse organisms facilitate the quest for new, nonstandard platforms to study various biological phenomena (Goldstein and King, 2016). We are entering the era in which reaching out to nontraditional models is easier than ever before, and for some research questions, it is not only very attractive but also inevitable. We are no longer limited to use of a single, well-establish platform for all research questions, but instead we can reach out to the animals which are most suitable for our specific scientific query. For instance, killifish would be a great choice to study ageing, tardigrades to explore resistance to complete desiccation or flatworms to investigate regeneration abilities (see Chapter 1).

This thesis demonstrates the potential of one of such nontraditional models. It also serves as an example on how to establish a new experimental platform. The gained experience emphasizes the importance of several steps. Generating a good quality genome and transcriptome assemblies is an essential first step, as it provides a reference for all future studies. In the course of this PhD, the genome and transcriptome of *M. lignano* underwent several improvements (Grudniewska et al., 2016; Wasik et al., 2015; Wudarski et al., 2017). Furthermore, there is no need for developing all tools from scratch. Often, methods used in other organisms will work in the new models. Despite that, technical challenges may emerge. It is important, therefore, to focus the efforts on the context and paradigms that are essential for a particular research question. Optimistically, however, the progress in understanding the biology of the study organism often leads to solution to earlier unsolvable problems.

In the coming years, we hope to see the further expansion of experimental inventory for *M. lignano*, which, in my opinion, may lead to groundbreaking discoveries.

References

Arbore, R., Sekii, K., Beisel, C., Ladurner, P., Berezikov, E., and Schärer, L. (2015). Positional RNA-Seq identifies candidate genes for phenotypic engineering of sexual traits. Front. Zool. *12*, 14.

Blasco, M., Finkel, T., and Serrano, M. (2007). The common biology of cancer and ageing. Nature *448*, 767–774.

Egger, B. (2008). Regeneration: Rewarding, but potentially risky. Birth Defects Res. Part C - Embryo Today Rev. *84*, 257–264.

Egger, B., Ladurner, P., Nimeth, K., Gschwentner, R., and Rieger, R. (2006). The regeneration capacity of the flatworm Macrostomum lignano - On repeated regeneration, rejuvenation, and the minimal size needed for regeneration. Dev. Genes Evol. *216*, 565–577.

Goldstein, B., and King, N. (2016). The Future of Cell Biology: Emerging Model Organisms. Trends Cell Biol. *26*, 818–824.

Grudniewska, M., Mouton, S., Simanov, D., Beltman, F., Grelling, M., de Mulder, K., Arindrarto, W., Weissert, P.M., van der Elst, S., and Berezikov, E. (2016). Transcriptional signatures of somatic neoblasts and germline cells in Macrostomum lignano. Elife *5*, 1–23.

Haranghy, L., and Balázs, A. (1964). Ageing and rejuvenation in planarians. Exp. Gerontol. *1*, 77–91.

Hashimoto, T., Horikawa, D.D., Saito, Y., Kuwahara, H., Kozuka-Hata, H., Shin-I, T., Minakuchi, Y., Ohishi, K., Motoyama, A., Aizu, T., et al. (2016). Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein. Nat. Commun. *7*, 12808.

Ladurner, Rieger, R., and Baguna, J. (2000). Spatial distribution and differentiation potential of stem cells in hatchlings and adults in the marine platyhelminth macrostomum sp.: a bromodeoxyuridine analysis. Dev. Biol. *226*, 231–241.

Laita, M., Laita, M., Mcteir, B., Mcteir, B., Pettersson, J., Pettersson, J., Ko, M., Ko, M., Spot, G.O., Spot, G.O., et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836–839.

Lange, C.S. (1968). A possible explanation in cellular terms of the physiological ageing of the planarian. Exp. Gerontol. *3*, 219–230.

Molinaro, A.M., and Pearson, B.J. (2016). In silico lineage tracing through single cell transcriptomics identifies a neural stem cell population in planarians. Genome Biol. *17*, 87.

De Mulder, K., Pfister, D., Kuales, G., Egger, B., Salvenmoser, W., Willems, M., Steger, J., Fauster, K., Micura, R., Borgonie, G., et al. (2009). Stem cells are differentially regulated during development, regeneration and homeostasis in flatworms. Dev. Biol. *334*, 198–212.

De Mulder, K., Kuales, G., Pfister, D., Egger, B., Seppi, T., Eichberger, P., Borgonie, G., and Ladurner, P. (2010). Potential of Macrostomum lignano to recover from γ-ray irradiation. Cell Tissue Res. *339*, 527–542.

Pfister, D., De Mulder, K., Philipp, I., Kuales, G., Hrouda, M., Eichberger, P., Borgonie, G., Hartenstein, V., and Ladurner, P. (2007). The exceptional stem cell system of Macrostomum lignano: screening for gene expression and studying cell proliferation by hydroxyurea treatment and irradiation. Front. Zool. *4*, 9.

Plusquin, M., De Mulder, K., Van Belleghem, F., Degheselle, O., Pirotte, N., Willems, M., Cuypers, A., Salvenmoser, W., Ladurner, P., Artois, T., et al. (2015). Toxic effects of cadmium on flatworm stem cell dynamics: A transcriptomic and ultrastructural elucidation of underlying mechanisms. Environ. Toxicol.

Rando, T. a (2006). Stem cells, ageing and the quest for immortality. Nature *441*, 1080–1086.

Rink, J.C. (2013). Stem cell systems and regeneration in planaria. Dev. Genes Evol. *223*, 67–84.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., et al. (2009). Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. Nature *459*, 262–265.

Signer, R.A.J., and Morrison, S.J. (2013). Mechanisms that regulate stem cell aging and life span. Cell Stem Cell *12*, 152–165.

Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell *126*, 663–676.

Tan, T.C.J., Rahman, R., Jaber-Hijazi, F., Felix, D.A., Chen, C., Louis, E.J., and Aboobaker, A. (2012). Telomere maintenance and telomerase activity are differentially regulated in asexual and sexual worms. Proc. Natl. Acad. Sci. U. S. A. *109*, 4209–4214.

Wagner, D.E., Wang, I.E., and Reddien, P.W. (2011). Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science *332*, 811–816.

Wasik, K., Gurtowski, J., Zhou, X., Ramos, O.M., Delás, M.J., Battistoni, G., El Demerdash, O., Falciatori, I., Vizoso, D.B., Smith, A.D., et al. (2015). Genome and transcriptome of the regeneration-competent flatworm, Macrostomum lignano. Proc. Natl. Acad. Sci. 201516718.

Williams, R.S., Moreno, P.I., Lowell, T. V, Jacobson, G.L., Singer, C., Shulmeister, J., Mclea, B., Blunier, T., Steig, E.J., Petit, J.R., et al. (1999). A Niche Maintaining Germ Line Stem Cells in the Drosophila Ovary. 1998–2001.

Van Wolfswinkel, J.C., Wagner, D.E., and Reddien, P.W. (2014). Single-cell analysis reveals functionally distinct classes within the planarian stem cell compartment. Cell Stem Cell *15*, 326–339.

Wudarski, J., Simanov, D., Ustyantsev, K., de Mulder, K., Grelling, M., Grudniewska, M., Beltman, F., Glazenburg, L., Demircan, T., Wunderer, J., et al. (2017). A platform for efficient transgenesis in Macrostomum lignano, a flatworm model organism for stem cell research. bioRxiv *151654*, 1–23.

Wurtzel, O., Cote, L.E., Poirier, A., Satija, R., Regev, A., and Reddien, P.W. (2015). A Generic and Cell-Type-Specific Wound Response Precedes Regeneration in Planarians. Dev. Cell *35*, 632–645.